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(54) Title: ANTISENSE GENE THERAPY FOR RNA VIRUSES (57) Abstract A vector and methods of inhibiting replication of an RNA virus which replicates in the cytoplasm of a cell in a subject, comprising administering to the cell of the subject a composition comprising a vector comprising a promoter functionally linked to a nucleic acid comprising a viral gene, wherein the nucleic acid is in antisense orientation relative to the promoter and wherein the cell is capable of promoting expression from the promoter, thereby transcribing the nucleic acid and inhibiting replication of the RNA virus in the subject. In particular, provided are vectors and methods for treating a respiratory syncytial virus infection.		

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ANTISENSE GENE THERAPY FOR RNA VIRUSES

This invention was made with government support under grant number A1 31900 awarded by NIAID. The government has certain rights in the invention.

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BACKGROUND OF THE INVENTION

Field of the Invention

10 The present invention is in the field of vectors for gene delivery, particularly for use in inhibition of replication of RNA viruses.

Background Art

Respiratory syncytial virus (RSV) is a leading cause of lower respiratory tract
15 illness in infants and young children. It is also associated with severe respiratory disease in the elderly (Garvie & Gray, 1980) and in adults with underlying cardiopulmonary or immune disease (Whimbey et al., 1995). The only approved drug for treatment of RSV infection is the nucleoside analogue ribavirin. Its widespread use has been called into question recently, after recent studies failed to demonstrate a salutary effect (Wheeler,
20 Wofford, and Turner, 1993).

Antisense oligonucleotides (oligos) have been extensively investigated as candidate antiviral agents (Cantin & Woolf, 1993; Stein & Cheng, 1993; Toulme, 1992; Cohen, 1991). Depending upon their method of introduction, oligos are found localized
25 in cytoplasmic vesicles (Zhao et al., 1993; Thierry & Ditschilo, 1992; Wagner et al., 1993) or in the nucleus (Mechti et al., 1991; Clarenc et al., 1993; Bennett et al., 1992). The limited information describing oligo inhibition of cytoplasmic viruses, such as RSV, may be a direct result of a difficulty in attaining sufficient levels of non-vesicle associated oligo in the cytoplasm (Gutierrez, et al., 1993, Raviprakash, et al., 1995).

30

The activity of antisense RNA has been demonstrated *in vitro* against many nuclear viruses, including mouse hepatitis virus (Maeda et al., 1995), polyoma virus (Ottavio et al., 1992), human cytomegalovirus (Monte et al., 1996), bovine leukemia

virus (Borisenko et al., 1992), moloney murine leukemia virus (Han et al., 1991), human immunodeficiency virus (Sczakiel and Pawlita, 1991; Vandendriessche et al., 1995), and human T-cell leukemia virus (von Ruden and Gilboa, 1989). However, effective antiviral agents against cytoplasmically replicating viruses, such as RNA viruses and RSV particularly, remain elusive.

The present invention addresses this great need for antiviral agents against cytoplasmically replicating viruses such as RSV by providing a composition comprising a vector that can inhibit replication of cytoplasmically replicating viruses. The vector comprises a promoter and a full length viral gene in antisense orientation relative to the promoter. Such a vector, though it can provide the cell with a viral template produced by the virus in the replication process, surprisingly works to inhibit replication of the virus. The inhibition is virus-specific and thus can be an effective treatment of a viral infection with minimal non-specific effects. Such a composition can have widespread use in many areas of the world to combat viral infections such as RSV infections.

SUMMARY OF THE INVENTION

The present invention provides a vector for inhibition of replication of an RNA virus that replicates in the cytoplasm of a cell, comprising a promoter functionally linked to a nucleic acid comprising a gene of the virus, wherein the nucleic acid is in antisense orientation relative to the promoter.

Accordingly, further provided by the present invention is a method of inhibiting replication of an RNA virus which replicates in the cytoplasm of a cell, comprising administering to the cell a composition comprising a vector comprising a promoter functionally linked to a nucleic acid comprising a viral gene, wherein the nucleic acid is in antisense orientation relative to the promoter and wherein the cell is capable of promoting expression from the promoter, thereby transcribing the nucleic acid and inhibiting replication of the RNA virus in the cell.

Additionally provided is a method of inhibiting replication of an RNA virus which replicates in the cytoplasm of a cell in a subject, comprising administering to the cell of the subject a composition comprising a vector comprising a promoter functionally linked to a nucleic acid comprising a viral gene, wherein the nucleic acid is in antisense orientation relative to the promoter and wherein the cell is capable of promoting expression from the promoter, thereby transcribing the nucleic acid and inhibiting replication of the RNA virus in the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Schematic representation of plasmid constructs used to transfect HEp-2 and 293 cells. (a) The RSV F gene was excised from pGEM/F and ligated into p636 in an antisense orientation to create plasmid p636/F. (b) F was excised from pGEMaF and ligated in pCEP to generate pCEP/F and pCEP/aF respectively. T7, T7 RNA polymerase promoter. CMV, human cytomegalovirus immediate early promoter. SV40, polyadenylation sequence from SV40. Hyg, hygromycin B phosphotransferase gene. H, *HindIII*. B, *BamHI*. K, *KpnI*. ori p, Epstein Barr virus origin of replication. EBNA, Epstein Barr virus nuclear antigen.

Figure 2 Inhibition of RSV plaque titer. Each unique cell line was infected with RSV as described in the Methods under Assay for Viral Yield. Inhibition was determined as a ratio of titer in experimental cell line compared to parental line control (e.g., cell lines derived from 293 cells were matched with 293 cells.)

Figure 3 Inhibition of RSV F protein expression in antisense F cell lines. (a) HEp-2 cell lines were infected with RSV for four days then examined for F protein via an F specific ELISA as described herein. (b) 293 cell lines were infected and examined for F protein as in (a). The OD in untransformed control cells was taken as 100%. Results are shown as % control OD. Bars indicate standard deviation and p values are shown.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations
5 therein will be apparent to those skilled in the art.

As used in the specification and in the claims, "a" can mean one or more, depending upon the context in which it is used.

RNA is normally found in both a nuclear and cytoplasmic location. Our work
10 has shown that, for RSV, antisense oligonucleotides have worked well at inhibiting translation of mRNA in a cell free system, but no effect could be detected *in vitro*. The present invention utilizes a full length viral gene, and the composition is demonstrated to have a specific inhibitory effect on replication of the virus to which it is directed.

The present invention provides a vector for inhibition of replication of an RNA
15 virus that replicates in the cytoplasm of a cell, comprising a promoter functionally linked to a nucleic acid comprising a gene of the virus, wherein the nucleic acid is in antisense orientation relative to the promoter. By an "RNA virus that replicates in the cytoplasm" is meant a virus that, in the cytoplasm, copies its RNA, makes the viral proteins necessary for packaging (including coat and envelope proteins, if present) a complete,
20 infectious virus, and assembles a virus capable of infection. Such an RNA virus essentially never enters the nucleus of the infected cell. In a preferred embodiment, the RNA virus is a virus that replicates primarily in the cytoplasm, that is, wherein at least 50%, more preferably at least 60%, 70%, 80%, 85%, 90%, or 95% of replication, including assembly events, takes place in the cytoplasm. In an even more preferred
25 embodiment, the RNA virus is a virus that replicates exclusively in the cytoplasm.

The vector can further comprise a polyA site functionally linked to the coding region. The vector can be a plasmid, a virus, an artificial chromosome, or any other vector. The vector can additionally contain any sequences useful for ultimately achieving expression of the vector in a cell into which the vector is transferred, as is
30 known in the art. For example, a plasmid vector can have an origin of replication, a selection gene, and the Epstein-Barr virus nuclear antigen. Thus, a plasmid vector can

be advantageously used for transient expression of the gene. A viral vector can additionally comprise its genes necessary to enter a cell but typically will be replication deficient. See, generally, *DNA cloning: A Practical Approach*, Volumes I and II, (Glover, D.M. ed., IRL Press Limited, Oxford, 1985).

- 5 By "functionally linked" is meant such that the promoter can promote expression of the heterologous viral gene, as is known in the art, such as appropriate orientation of the promoter relative to the heterologous viral nucleic acid. Furthermore, the viral nucleic acid preferably has all appropriate sequences for expression of the nucleic acid, as known in the art. It is preferable that the promoter be a relatively strong
10 promoter. Furthermore, the promoter can be selected based upon the cell into which the vector is to be transferred, that is, a promoter that works well in that cell. Furthermore, an inducible promoter can be used, so that expression of the vector can be controlled; in time or in a specific organ or tissue. Known strong promoters such as SV40 or the inducible metallothionein promoter can be used. Additional examples of promoters
15 include promoters derived from action genes, immunoglobulin genes, cytomegalovirus (CMV), adenovirus, bovine papilloma virus, adenoviral promoters, such as the adenoviral major late promoter, an inducible heat shock promoter, respiratory syncytial virus, Rous sarcomas virus (RSV), etc.

- The gene is substantially full length. While the gene can be a genomic sequence,
20 it is preferred that the gene be a cDNA sequence. As used herein, a "full length" gene encompasses a full length cDNA. "Substantially" full length can be exemplified by a nucleic acid having at least 80%, more preferably 90% of the genomic or cDNA sequence present, such as 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the gene. Any nucleotides missing from the nucleic acid can be at either the 5'
25 end, the 3' end or interior within the gene; additionally, missing nucleotides can be scattered throughout the gene, so long as the ultimate antisense mRNA made in the cell transfected with the vector can selectively hybridize to the target viral RNA in the conditions in the cytoplasm of the cell. However, the gene should be of a length such that the antisense mRNA encoded by the vector can selectively hybridize to the target
30 viral RNA in the conditions in the cytoplasm of the cell; additionally, the gene must be able to inhibit replication of the virus when administered to a cell infected with the virus.

Additionally, conservative modifications from the viral sequence can be made, as long as the ultimate antisense mRNA made in the cell transfected with the vector can selectively hybridize to the target viral RNA in the conditions in the cytoplasm of the cell. This selective hybridization can readily be determined *in vitro* by following the methods exemplified herein and determining whether the modified antisense molecule causes inhibition of the virus. Typical modifications are known in the art.

To make a vector comprising any selected viral gene in antisense orientation, one can simply use the RNA genome to synthesize a cDNA copy of the genome. This cDNA copy is then inserted into a selected carrier plasmid or virus or artificial chromosome in antisense relative to a promoter present in the final vector. Such methods are known in the art (*see, e.g.,* Sambrook et al.)

Additionally provided by the present invention is a cell containing a vector of this invention. Such cells can be a cell in which the vector can replicate. Such cells can be, for example, mammalian cells, yeast cells or bacterial cells.

15

The present invention additionally provides a method of inhibiting expression and/or replication of a virus that replicates in the cytoplasm of a cell. The invention particularly provides a method of inhibiting expression and/or replication of an RNA virus. The method comprises administering to a cell, prior to or after infection, a composition comprising a vector comprising a promoter functionally linked to a nucleic acid comprising a viral gene, wherein the nucleic acid is in antisense orientation relative to the promoter and wherein the cell is capable of promoting expression from the promoter, thereby transcribing the nucleic acid and inhibiting replication of the RNA virus in the cell. The vector can also comprise a polyA site functionally linked to the coding sequences. By administering the vector to the cell, the gene product will ultimately be transcribed by the cell to make an mRNA. The mRNA will be shuttled by the cell out of the nucleus into the cytoplasm. The mRNA, which is an antisense molecule relative to a selected strand of the virus, then can hybridize to its target RNA of the virus. The vector can be, for example, a plasmid vector, a viral vector, a cosmid vector or an artificial chromosome vector.

30

The present invention further provides a method of inhibiting replication of an RNA virus which replicates in the cytoplasm of a cell in a subject, comprising administering to the cell of the subject a composition comprising a vector comprising a promoter functionally linked to a nucleic acid comprising a viral gene, wherein the

5 nucleic acid is in antisense orientation relative to the promoter and wherein the cell is capable of promoting expression from the promoter, thereby transcribing the nucleic acid and inhibiting replication of the RNA virus in the subject. The vector can be, for example, a plasmid vector, a viral vector, a cosmid vector or an artificial chromosome vector. The composition can be administered in an amount sufficient to reduce levels of

10 virus in the cells of the subject, and preferably thus in an amount sufficient to treat a disease associated with the viral infection. By treating is included reduction in viral titer, reduction in symptoms caused by the viral infection, reduction in time length of infection, reduction in time length of symptoms, and other effects of treatment as known in the art.

15 The present method can be used with any RNA virus that replicates in the cytoplasm of its host cell. The present invention provides the antisense construct to the cell as a plasmid or other vector nucleic acid. The cell then produces the antisense mRNA molecules, which are carried out into the cytoplasm. Thus the antisense molecules are provided to the cytoplasm to perform their inhibitory function. The RNA

20 virus can be either a positive-sense RNA virus or a negative-sense RNA virus. Furthermore, in the embodiment of this invention provided here, the mRNA produced by the construct in the cell lacks translation initiation codons.

The present invention can be used to inhibit expression and replication of any

25 virus that replicates in the cytoplasm. RNA viruses typically replicate in the cytoplasm. This method can be used with any RNA virus that replicates primarily in the cytoplasm, and preferably an RNA virus that replicates exclusively in the cytoplasm. It is not used to inhibit replication of RNA viruses having "slow" infection diseases, such as the disease produced by a slow measles virus infection; rather, it is used for RNA viruses

30 actively replicating. Aphthovirus can also be excepted from RNA viruses for which the present invention is used. Examples of RNA viruses, for which the present invention

can be utilized, include poliovirus, coxsackieviruses, enteroviruses (causes diarrhea), hepatitis A, rhinovirus (common cold), caliciviridae (causes encephalitis) flaviviridae (causes yellow fever, dengue fever, encephalitis), influenza, mumps, rabies, rotavirus (causes diarrhea).

5

Other cytoplasmically replicating RNA viruses include Paramyxoviruses (e.g., Sendai virus, parainfluenza viruses (such as parainfluenza 1 virus, parainfluenza 2 virus, parainfluenza 3 virus [human, cattle], parainfluenza 4 virus, Newcastle disease virus), and mumps virus); Morbilliviruses (e.g., rinderpest virus [cattle], canine distemper virus, 10 peste-des-petits-ruminants virus of sheep and goat, excepting measles virus); Pneumovirus (e.g., respiratory syncytial virus [human, bovine], pneumonia virus [mice]). The preceding list includes negative sense RNA viruses having F protein. However, any RNA virus which replicates in the cytoplasm can be inhibited by this method (see, e.g., *Fundamental Virology*).

15 Any viral gene can be used in the present vectors and in the present method. In a preferred embodiment, the gene is a viral gene necessary for replication of the virus, i.e., necessary for the virus to assemble and cause infection. For example, the gene encoding the fusion, or F protein, that several enveloped RNA viruses produce, can be used.

F protein is a major factor in fusion of the virus lipoprotein envelope with the 20 lipoprotein surface membrane of the host cell. F proteins are synthesized as active precursors (F_0) (comprising about 540-580 amino acids) which gain the fusion function by a posttranslational cleavage process that is mediated by cellular trypsin-like enzymes. The larger cleavage product, F_1 , has a hydrophobic sequence at the amino terminus, which serves as the site of insertion of the F protein into the membrane of a host cell.

25 Other RSV genes that can be used in the present invention include major nucleocapsid protein (N), nucleocapsid phosphoprotein (P), large nucleocapsid protein (L), large glycoprotein (G), nonglycolated matrix protein (M), a second matrix protein (22K), two nonstructural proteins (1B and 1C), and small hydrophobic protein (1A).

Another protein for which an antisense construct could be used in the present 30 method can be the influenza virus HA protein (fusion and attachment functions).

Furthermore, a major surface glycoprotein of lentiviruses, such as HIV-1, HIV-2, SIV-3, which has homologies to F protein, can be used.

The gene which can be utilized is not limited to protein in the envelope, particularly for non-enveloped RNA viruses. Other genes which can be utilized in this invention include, but are not limited to, a polymerase, *i.e.* an RNA polymerase, such as L polymerase, or an early gene. Additionally useful would be a gene expressed at low amounts, such as a polymerase. Using a gene expressed early in the viral cycle could be beneficial because its inhibition by the antisense molecule would stop replication of the virus at an early stage. Using a gene expressed at low levels can be beneficial because lower amounts of antisense mRNA could be needed to inhibit viral replication. Note, however, that low amounts of F protein antisense mRNA appear to achieve high levels of inhibition, as seen in the examples herein.

Other viral genes which can be used in the present vector for use in inhibiting replication of a virus include any capsid protein from viruses of the Picornoviridae, Calciviridae, Togaviridae, Flaviviridae, Coronaviridae, Paramyxoviridae, Rhabdoviridae, Orthomyxoviridae, Bunyaviridae, Arenaviridae.

The present methods can be used to treat a viral infection by a virus that replicates in the cytoplasm. Treating can include eliminating the virus from the cells of the subject and/or reducing the effects of the viral infection. Thus the present methods can be used to treat diseases caused by such a viral infection. The present invention can also be used to prevent establishment of a viral infection in cells, particularly in cells in a subject. Diseases associated with specific RNA viruses are known in the art. Diseases associated with specific RNA viruses and treated by the present invention can include, for example, diarrhea, hepatitis, the common cold, encephalitis, yellow fever, dengue fever, influenza, measles, mumps, rabies. Additionally, treatment of disease conditions including, for example, AIDS; upper and lower respiratory tract infections; rubella; polio; feline AIDS; and bovine AIDS are contemplated by the invention.

The vector can be administered in any of several means, which can be selected according to the vector utilized, the organ or tissue, if any, to be targeted, and the

characteristics of the subject. The present vectors need not be specifically targeted to any particular cell type, organ or tissue, since the expression of the vector in a cell not infected by the corresponding virus, will have no adverse effect on the cell, since this method does not interfere with a cellular function. Therefore, the vectors, if desired in a pharmaceutically acceptable carrier such as physiological saline, can be administered systemically, such as intravenously, intraarterially, orally, parenterally, subcutaneously. The vector can also be administered by direct injection into an organ or by injection into the blood vessel supplying a target tissue. For an infection of cells of the lungs or trachea, such as by RSV, it can be administered intratracheally or by inhalation or aerosolization. The vectors can additionally be administered topically, transdermally, etc.

For plasmid or artificial chromosome vectors, the plasmid can be administered in a composition. For example, the composition can comprise other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc. Furthermore, the composition can comprise, in addition to the vector, lipids such as liposomes, such as cationic liposomes (e.g., DOTMA, DOPE, DC-cholesterol) or anionic liposomes. Liposomes can further comprise proteins to facilitate targeting a particular cell, if desired. Administration of a composition comprising a vector and a cationic liposome can be administered to the blood afferent to a target organ or inhaled into the respiratory tract to target cells of the respiratory tract. Regarding liposomes, see, e.g., Brigham et al. *Am. J. Resp. Cell. Mol. Biol.* 1:95-100 (1989); Felgner et al. *Proc. Natl. Acad. Sci USA* 84:7413-7417 (1987); U.S. Pat. No. 4,897,355.

For a viral vector, the composition can comprise a pharmaceutically acceptable carrier such as phosphate buffered saline or saline. The viral vector can be selected according to the target cell, as known in the art. For example, adenoviral vectors, in particular replication-deficient adenoviral vectors, can be utilized to target any of a number of cells, because of its broad host range. Many other viral vectors are available, and their target cells known.

Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection or aerosolization, or as emulsions. Typically, a dosage will be about 0.5 µg/kg to about 1.0 µg/kg of DNA. In general, a useful dosage can be a typical dosage used in administration of plasmid DNA/cationic liposomes to prevent acute lung injury to endotoxin. Dosages will depend upon the mode of administration, the disease or condition to be treated, the target organ, tissue or cells, and the individual subject's condition, but will be that dosage typical for and used in administration of other plasmid or viral vectors (see e.g., U.S. Pat. No. 4,897,355). Often a single dose can be sufficient; however, the dose can be repeated if desirable.

The vectors of this invention can also be administered to a cell *ex vivo* and the cell then transferred into a subject. In particular, a cell, tissue or organ can be removed from a subject, transfected with a vector, and returned to the subject. For example, one could harvest a subject's T cells, transfect them with a vector having an HIV antisense sequence, and return the cells to the patient to treat an HIV infection or to render the subject resistant to HIV infection. In general, for such an *ex vivo* administration, cells are isolated from a subject by standard means according to the cell type and placed in appropriate culture medium, again according to cell type (see, e.g., ATCC catalog). Vector is then contacted with the cells as appropriate for the vector type, and the vector is allowed to transfer into the cells. Cells can then be transplanted back into the subject's body, again by means standard for the cell type and tissue (e.g., in general, U.S. Patent No. 5,399,346). If desired, prior to transplantation, the cells can be studied for degree of transfection by the selected vector, such as a selected virus or a selected transfer medium for a plasmid-type vector, by known detection means and as described herein. Cells for *ex vivo* transfection followed by transplantation into a subject can be selected from those listed herein, or can be any other selected cell.

Subjects can be any subject selected, particularly a mammalian subject, such as human, primate, such as monkey or chimpanzee, horse, cow, sheep, goat, rabbit, guinea pig, rat, mouse.

Statement Concerning Utility

The present invention has applicability in treating an infection by any RNA virus that replicates in the cytoplasm, and particularly RNA viruses that replicate exclusively in the cytoplasm. The major problem with previous antiviral agents is their poor
5 specificity. Since the virus takes over the cell machinery in order to replicate, often a therapy aimed at killing the virus often kills the cell, oligonucleotide therapy (*i.e.*, administering a small strand of RNA or DNA that is antisense for the viral gene) has the advantage of being toxic specifically to the virus and not the cell. The present invention also has this advantage, but the two technologies are critically different in that in the
10 present invention, the full length antisense viral gene is expressed. The advantage of this is the prolonged half-life as well as increased efficacy. Oligonucleotide half-life is in the order of hours; transferred plasmid half-life is measured in days. Furthermore, expressing the full length antisense gene is tremendously more efficient *in vitro* in inhibiting viral replication than delivering the oligonucleotide. The present vectors can
15 also be utilized to further analyze infection stages and mechanisms used by a selected virus.

EXAMPLES

Cells and Viruses HEp-2 cells, originally obtained from the American Type
20 Culture Collection (ATCC), were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). 293 cells were maintained in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% FCS. Stocks of RSV, strain A2, and human parainfluenza virus type 3 (HPIV-3) were routinely grown in HEp-2 cells.

25

Vector construction The RSV fusion protein gene (F) was originally cloned via reverse transcription (RT) of total RNA from RSV infected cells followed by
polymerase chain reaction (PCR) amplification as in Heminway et al. (1994). RT-PCR products were run on 1% agarose gel and visualized by ethidium bromide staining. The
30 F gene fragment was excised and purified as described in Sambrook et al. (1989) and directionally cloned into the *Bam*HI and *Hind*III restriction sites of pGEM-3 (Promega)

to yield pGEM/F. This clone was verified by sequencing in its entirety. Plasmid pGEMaF was constructed using pGEM-F as template in a PCR to amplify the F insert using a primer pair designed to add restriction sequences for the purpose of subcloning. The PCR product was cloned as described above into corresponding restriction
5 endonuclease sites in pGEM-3 to make pGEMaF. F and aF were identical except for the reversal of the location of the *Bam*HI and *Hind*III restriction sites.

p636/aF The F gene was subcloned in the antisense orientation into vector p636 (gift of B. Sugden, Univ. of Wisconsin) by *Bam*HI and *Hind*III digestion of pGEM/F,
10 isolation of the *Bam*HI /*Hind*III F gene fragment, and subsequent ligation into the corresponding sites in p636. The vector contains a mammalian expression cassette consisting of a human cytomegalovirus (CMV) immediate early promoter, *Bam*HI and *Hind*III restriction sequences for cloning, an SV40 polyadenylation sequence for efficient termination and polyadenylation of the transcript, and the hygromycin B
15 phosphotransferase gene.

pCEP/F and pCEP/aF The F gene was subcloned into vector pCEP-4 by *Hind*III and *Kpn*I digestion of pGEMaF, isolation of the *Hind*III /*Kpn*I F gene fragment and subsequent ligation into the corresponding sites in pCEP-4 to generate pCHPaF.
20 The sense counterpart, pCEP/F, was constructed by *Bam*HI and *Hind*III digestion of pGEMaF, isolation of the F gene fragment and ligation into the corresponding sites in pCEP-4. This vector contains the same mammalian expression cassette as in p636. In addition, the Epstein-Barr Virus (EBV) origin of replication (*ori* p) and the Epstein-Barr Virus Nuclear Antigen (EBNA-I) are encoded so the plasmid can be maintained and
25 replicate episomally in the nucleus. All clones were purified by CsCl banding.

Selection of stably transfected cell lines

293 cells Transfection medium consisting of 2µg p636/aF, 0.1µg pSV2Neo and 24 nmol of the cationic lipid DC-cholesterol diluted to 0.5 ml in Hanks balanced salt
30 solution (HBSS) was prepared and incubated at 37 C for 10 min. 293 cells in 12 well plates, (~1x10⁶ cells/well), were rinsed twice with HBSS followed by an addition of 500

μl/ well transfection media. Incubation proceeded for 4 hours at 37°C followed by replacement of medium with 1 ml MEM + 10% FCS (MEM-10). At 48 hours post transfection (p.t.), cells were split 1:4 into MEM-10 containing 500 μg/ml G418 (Sigma) and maintained until the formation of colonies which were then either isolated and expanded from single colonies or pooled from a group of colonies for use in the described experiments.

Hep-2 cells Plasmids pCEP/F and pCEP/aF were complexed with Lipofectin (Gibco-BRL) in Opti-MEM (Gibco-BRL) according to the manufacturers recommendation, and used to transfect HEp-2 cells as follows; HEp-2 cells, ~70% confluent in one 6 well plate, were rinsed twice with 2 mls serum free Dulbecco's Modified Eagle Media (DMEM) and 500 μl transfection media was placed on each well and incubated at 37 C for 1 hour. Then, 2 mls DMEM with 10% FCS (DMEM-10) was added and incubation proceeded for 24 hours at 37°C. The transfection media was replaced with 2 mls DMEM-10 containing 350 μg/ ml Hygromycin B the next day and every two days thereafter. Colonies were isolated on day 17 p.t. and expanded for use in the described experiments.

ELISA assay for viral protein At 4 days post infection (p.i.), replicate cultures of RSV infected cells were washed twice with phosphate buffered saline (PBS) and fixed by addition of 1 ml 50:50 acetone/methanol for 20 min at room temperature. The fixative was removed, the plate air dried, and a blocking solution, (5% non-fat dry milk in PBS/0.1% Tween (PBS/Tw)), placed in each well for 30 min. at 37°C. This was replaced by 0.5 mls of blocking solution per well containing 1:10,000 dilution of G11C6, an anti-RSV F monoclonal antibody (Baumann et al., 1987). Binding occurred at 37°C for 2 hrs. Wells were then washed four times with PBS/Tw, followed by addition and incubation of 0.5 mls blocking solution containing 1:3000 dilution of horse radish peroxidase conjugated goat anti-mouse antibody (Sigma) for 90 min. at 37°C. The wells were washed four times with PBS/Tw and twice with PBS. The plates were developed by adding 0.5 ml of a 0.05 M phosphate-citrate buffer containing 1mg/ ml o-phenylenediamine dihydrochloride (Sigma) and incubation in the dark at 37°C until

sufficient color was visible. An equal volume of 2.5 M H₂SO₄ was added to stop the reaction. The optical density of 100 µl aliquot of each sample was measured on a Dynatech MR600 microplate reader at 490 nm. Each assay was performed in triplicate and results compared to a control. The students t test was used to compare mean
5 absorbance values. A p value <0.05 was considered significant.

Assay for viral yield Cell lines, 100% confluent in 24 well plates, were infected with RSV at a multiplicity of infection (m.o.i.) of ~0.01. At 4 days p.i., cells were frozen at -80°C and thawed once. Lysates were clarified by centrifugation at 12000 x g for 5 min,
10 and virus containing supernatants were used to determine RSV titer by plaque assay on HEp-2 cells. Briefly, HEp-2 cells in 24 well plates were washed twice in phosphate buffered saline (PBS) followed by absorption of 0.1 ml of virus dilution for 1 hr at 37°C. Inoculum was replaced with 0.5 mls of an equal mixture of 2X MEM containing 4% FCS and 1.8% (w/v) agarose. Incubation proceeded at 37°C for 6 days while
15 plaques formed. Cells were then fixed with a 2% glutaraldehyde solution in PBS and stained with 1% crystal violet solution in 20% ethanol to visualize plaques.

Plaque morphology Plaque assays were carried out directly on antisense and control cell lines essentially as described above. Plaques were visualized by crystal violet
20 staining and examined by microscopy.

Detection of cDNA sequences in transformed cell lines Antisense or control cells (~1x10⁵ cells) were trypsinized, pelleted at 12000 x g for 30 seconds, resuspended in 50 µl water and boiled for 5 min. Following centrifugation for 2 min. at 12,000 x g,
25 supernates were removed and extracted once with phenol/chloroform, once with chloroform, and then ethanol precipitated. Pellets were resuspended in 20 µl water and a 5 µl aliquot of each was used in a PCR with the F gene specific primers F1 and F2 described in vector construction. Cycling parameters were as follows: 1 min. at 94°C, 35 cycles of 1 min. at 94°C followed by 1 min. at 52°C, and then ending with a 7 min.
30 extension at 52°C. Products were run on 1% agarose gel and visualized by ethidium bromide staining.

Full length F gene and the corresponding transcript was detected in antisense F cell lines as follows. A 1.8 Kb fragment was amplified using DNA isolated from 293 antisense F clones Pool 1, Pool 4, D4, F9, and C10. F DNA was to detected in untransformed 293 cells, or in the two antisense F clones D7 and Pool 2. As a PCR
5 amplification control, 293 DNA was doped with pGEM/F before amplification. A 1.8 Kb fragment was amplified using DNA isolated from the HEp-2 antisense F clones aF-B, aF-C, and aF-D. F DNA was not detected in untransformed HEp-2 cells or cells transformed with the sense F construct pCEP/F. To control for PCR inhibitors in the crude DNA preps, HEp-2 DNA was doped with pGEM/F and subjected to PCR
10 amplification. Total RNA isolated from the indicated cell lines was subjected to RT-PCR designed to amplify a 700 bp fragment of antisense F RNA as described herein.

Detection of antisense RNA TRI-Reagent (Molecular Research Center, Inc.) was used to extract total RNA from antisense or control cells ($\sim 2.5 \times 10^6$ cells) in 3.5 cm
15 dishes according to the manufacturer's instructions. An additional digestion with RNase free DNase 1 (Boehringer) was required to rid the RNA prep of contaminating DNA. For northern blot detection of antisense RNA, digoxigenin labeled RNA probes were prepared by *in vitro* transcription of pGEM/F according to the manufacturer's instructions (Boehringer). For detection via RT-PCR, the RNA (2 μ g) was reverse
20 transcribed into single stranded cDNA and was then subjected to PCR in a final volume of 100 μ l.

RESULTS

Construction of antisense vectors and cell lines

p636/anti-F. The RSV F gene from pGEM/F was subcloned in an antisense
25 orientation with respect to the promoter in p636 to yield p636/aF as shown in Fig. 1a. Transcription of p636/aF will produce RNA complementary in sequence to RSV F mRNA. This construct was used in conjunction with pSV2*Neo*, which expresses the neomycin resistance gene, to transfect 293 cells and positive transformants were selected with G418 as described herein. Sixteen separate populations arising from both
30 single clones and pooled clones were generated.

pCEP/aF and pCEP/F. The RSV G gene from pGEMaF was subcloned in both a sense and antisense orientation with respect to the CMV promoter into vector pCEP to yield pCEP/aF and pCEP/F (Fig. 1b) and used to transform HEp-2 cells. Positive transfectants were selected with hygromycin B as described herein and four clones were isolated and expanded.

Detection of the RSV F gene in stably transfected cell lines

All antibiotic resistant clones obtained were screened for the delivered transgene in order to correlate its presence with the cell lines' biologic properties. Total DNA was isolated and subjected to PCR with the F gene specific primers F1 and F2 as described herein. These primers were complementary to the ends of the transfected F gene and will thus amplify full length F. Cells in which the F gene is truncated, rearranged, or deleted will not give a positive signal in this PCR. Twelve 293 antisense F clones were examined. Five show the presence of a 1.8 Kb band after PCR amplification, corresponding to the full length F gene while two were negative. Clones F9 and C10 were selected for further study to determine the effect of antisense F sequences on RSV infection in 293 cells and clone D7 was selected as a transformed control. The four HEp-2 cell clones were also examined, of which three were positive for the full length F gene and one negative. The clones anti-F B, anti-F C, and anti-F D were studied to determine the effect of antisense F sequences in RSV infected Hep-2 cells while pCEP/F was used as a transformed control.

Detection of antisense RNA in stably transfected cell lines

We next analyzed the cells for the presence of transcripts of the delivered transgenes. Briefly, total RNA was isolated from each clone and 10 µg was used in an RNA dot blot procedure. Antisense RNA could not be detected in any clone by this method (data not shown). We then tried a more sensitive assay. An RT-PCR designed to amplify antisense F RNA was performed. A 700 bp fragment amplified from antisense F RNA in clone C10 was detected. The absence of a band in the C10 RT(-) control indicates that amplification was truly from antisense RNA and not contaminating

DNA. C10 was the only 293 clone with detectable amounts of antisense RNA.

Antisense RNA could not be detected in any HEp-2 clones using either method.

RSV F protein levels are reduced in antisense F cell lines

5 Cellular antisense F RNA should interfere with F mRNA translation and thus decrease the amount of F protein synthesized. To investigate this, control and antisense cell lines were infected with RSV for 4 days, then fixed and examined for total F protein expression via an F specific ELISA. Table 1 shows the amount of F protein detected in antisense F cell lines relative to that in the untransformed control cells.

10

Table 1

Cell Line	% Inhibition ELISA OD	P (compared to control)
aF-B	70	0.002
15 aF-C	7	0.016
aF-D	42	0.014
pCEP/F	62	0.001
F9	50	0.002
C10	90	0.001
20 D7	-10	NS

All three HEp-2 antisense F clones had decreased levels of F protein at four days after infection. Surprisingly, the sense F control, pCEP/F, also demonstrated a significant reduction in F expression. Of the three selected 293 antisense F cell lines, clones F9 and C10 showed significant decreases in F protein levels compared to the parent 293 cells, while the control clone D7 did not.

RSV Yield is decreased in antisense F cell lines

30 Interference with the production of a viral protein may be expected to delay or decrease virus multiplication. We observed such a decrease in antisense F cell lines that

correlated with the presence of antisense F sequences. Antisense and control cell lines were infected with RSV at an m.o.i. of 0.01. Four days p.i., the cells were harvested by freeze/thawing once. Clarified supernates were used in a plaque assay on HEp-2 cell monolayers. Results of viral yield experiments are summarized in Figure 3. Viral multiplication was decreased in anti-F B, anti-F C and anti-F D clones as indicated by the decrease in RSV infectious particle yield compared to HEp-2 cells. In agreement with an antisense mechanism of inhibition, no decrease in RSV replication on the sense F cell line pCEP/F was observed. The most dramatic decrease in viral multiplication was seen in the 293 antisense clones F9 and C10 with almost a 2 log reduction in RSV yield. Clone D7, negative for antisense F sequences, showed no decrease in virus multiplication.

Inhibition of syncytia formation

Inhibition of viral CPE would be an indication of interference in RSV multiplication. RSV plaque formation in HEp-2 cells occurs when the F protein mediates the formation of syncytia, which then disintegrate leaving a hole in the monolayer. To investigate whether syncytia formation was inhibited in antisense F cells, RSV was plaqued in anti-F B, anti-F C, anti-F D, pCEP/F and the parent HEp-2 cell line. Normal, syncytial plaques formed in the parent cell line HEp-2 as well as the sense F line pCEP/F. Non-syncytial plaques were found only in antisense F cell lines indicating an alteration in F protein function.

Antisense inhibition is specific for RSV

To determine if the inhibitory effect seen in the antisense F cell lines was specific for RSV, two antisense and two control cell lines were infected with the related paramyxovirus HPIV-3 and infectious particle yield was determined by plaque assay on HEp-2 cells. The replication of HPIV-3 was not inhibited in the two cell lines most inhibitory to RSV, anti-F B and C10, indicating the inhibitory effect on RSV multiplication is specific.

In these studies, we utilized plasmid vectors to express antisense RNA in two RSV susceptible cell lines. The pCEP/aF and pCEP/F vectors, which are maintained episomally in the nucleus, were used to transfect HEp-2 cells and were selected by resistance to hygromycin B. The p636aF construct, which integrates into the host cell genome, was used to transform 293 cells and was selected by resistance to geneticin. Although different express plasmids, cell lines, nuclear environments and selection drugs were employed, clones were identified from each system that were inhibitory to both viral yield and expression of F protein. Thus, the inhibitory effects seen with this anti-gene strategy appear to be generally applicable and not limited to cell line or integration state of the vector.

The decrease of RSV yield in these cell lines correlates with the presence of the antisense sequences. All clones containing the full length transgene showed a decrease in RSV yield, whereas the transformed controls, clones D7 and pCEP/F, did not (Figure 3). Interestingly, the only clone with a detectable amount of antisense RNA, C10, showed the greatest inhibition in both F protein synthesis and yield of infectious virus. Our other clones, in which antisense RNA was not detected, must still contain a sufficient amount of antisense RNA to exert an inhibitory effect on RSV.

Enzymes reported responsible for degradation of antisense sense RNA duplexes such as double strand specific RNases (Strickland et al., 1988) and RNA unwindase activities (Rebagliati and Melton, 1987) are present almost exclusively in the nucleus (Wagner et al., 1990). According to the model of Cornelissen (1989), antisense inhibition occurs most effectively in the nucleus. The current understanding of antisense action in the cytoplasm is that of a transient sense:antisense interaction that reduces the efficiency of translation, but does not effect mRNA levels (Cornelissen & Vandewiele, 1989). How virtually undetectable amounts of antisense RNA can cause significant inhibition in the cytoplasm may be better understood by taking into account the probable site of action. Negative stranded viruses, such as RSV, produce sense RNA in the form of a series of sub-genomic mRNAs coding for the viral proteins, and also as genome length replicative intermediates (RI). An antisense interaction could occur at either or both places. In agreement with the conclusions of Koschel et al., (1995) concerning

antisense inhibition of measles virus, we feel that interference with RI RNA is the more probable mechanism. The RI RNA is of much lower abundance in infected cells than RSV mRNA, and, because of its role as a template for synthesis of the viral genome, an antisense interaction here is more likely to decrease viral yield by interfering with
5 replication. Indirect evidence supporting this latter possibility is seen in our data by comparing activity of the control cells, pCEP/F, with the antisense cell counterparts. Though F protein synthesis is diminished in pCEP/F cells, RSV yield is not. Inhibition of F protein translation may not be a primary mechanism of antisense inhibition in this system.

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Plaque assay on antisense and sense cell lines, however, suggest that an antisense interaction is occurring at both the RI and F mRNAs. Plaque assay on the antisense F cell lines revealed non-syncytial plaques that were smaller than the normal syncytial plaque phenotype. If interference occurred at RI RNA only, syncytial plaques would
15 still be expected, even though they may be smaller than wild type due to a decrease in viral replication. The absence of fusogenic plaques argues for a corresponding inhibition in F protein function that is most likely brought about by lower levels of F protein. The decrease in F protein must only be sufficient to inhibit fusion if there is a concomitant decrease in RSV replication. That may be the reason for a normal plaque phenotype
20 seen in pCEP/F cells despite ELISA results showing a decrease in F protein.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this
25 invention pertains.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that
30 they are included in the accompanying claims.

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What is claimed is:

1. A vector for inhibition of replication of an RNA virus that replicates in the cytoplasm of a cell, comprising a promoter functionally linked to a nucleic acid comprising a gene of the virus, wherein the nucleic acid is in antisense orientation relative to the promoter.
2. The vector of claim 1, wherein the vector is a plasmid vector.
3. The vector of claim 1, wherein the promoter is cytomegalovirus promoter.
4. The vector of claim 1, wherein the virus is respiratory syncytial virus (RSV) and the nucleic acid is an RSV gene.
5. The vector of claim 1, wherein the nucleic acid encodes an RSV viral protein.
6. The vector of claim 5, wherein the nucleic acid encodes an RSV viral protein necessary for replication of the RSV virus.
7. The vector of claim 1, wherein the nucleic acid encodes RSV F protein.
8. The vector of claim 1, wherein the vector further comprises a polyadenylation site functionally linked to the 3' end of the nucleic acid.
9. The vector of claim 2, wherein the vector further comprises an origin of replication.
10. The vector of claim 2, wherein the vector further comprises a nucleic acid encoding Epstein -Barr Virus Nuclear Antigen.
11. A composition comprising the vector of claim 1.
12. The composition of claim 11, wherein the composition further comprises a pharmaceutically acceptable carrier.
13. A cell containing the vector of claim 1.
14. A method of inhibiting replication of an RNA virus which replicates in the cytoplasm of a cell, comprising administering to the cell a composition comprising a vector comprising a promoter functionally linked to a nucleic acid comprising a viral gene, wherein the nucleic acid is in antisense orientation relative to the promoter and wherein the cell is capable of promoting expression from the promoter, thereby transcribing the nucleic acid and inhibiting replication of the RNA virus in the cell.
15. The method of claim 14, wherein the RNA virus is respiratory syncytial virus and the viral gene is a respiratory syncytial virus gene.

16. The method of claim 15, wherein the nucleic acid encodes an RSV viral protein necessary for replication of the RSV virus.
17. The method of claim 14, wherein the nucleic acid encodes a viral protein.
18. The method of claim 14, wherein the nucleic acid encodes RSV F protein.
19. The method of claim 14, wherein the vector further comprises a polyadenylation site functionally linked to the 3' end of the nucleic acid.
20. The method of claim 14, wherein the promoter is cytomegalovirus promoter.
21. The method of claim 14, wherein the RNA virus is a negative-strand virus.
22. The method of claim 14, wherein the RNA virus is a positive-strand virus.
23. The method of claim 14, wherein the vector is a plasmid vector.
24. The method of claim 14, wherein the composition further comprises a pharmaceutically acceptable carrier.
25. A method of inhibiting replication of an RNA virus which replicates in the cytoplasm of a cell in a subject, comprising administering to the cell of the subject a composition comprising a vector comprising a promoter functionally linked to a nucleic acid comprising a viral gene, wherein the nucleic acid is in antisense orientation relative to the promoter and wherein the cell is capable of promoting expression from the promoter, thereby transcribing the nucleic acid and inhibiting replication of the RNA virus in the subject.
26. The method of claim 25, wherein the RNA virus is respiratory syncytial virus and the viral gene is a respiratory syncytial virus gene.
27. The method of claim 26, wherein the nucleic acid encodes an RSV viral protein necessary for replication of the RSV virus.
28. The method of claim 25, wherein the nucleic acid encodes a viral protein.
29. The method of claim 25, wherein the nucleic acid encodes RSV F protein.
30. The method of claim 25, wherein the vector further comprises a polyadenylation site functionally linked to the 3' end of the nucleic acid.
31. The method of claim 25, wherein the promoter is cytomegalovirus promoter.
32. The method of claim 25, wherein the RNA virus is a negative-strand virus.
33. The method of claim 25, wherein the RNA virus is a positive-strand virus.
34. The method of claim 25, wherein the vector is a plasmid vector.

35. The method of claim 25, wherein the composition further comprises a pharmaceutically acceptable carrier.
36. The method of claim 26, wherein an amount of the composition sufficient to reduce an RSV infection in the subject is administered.

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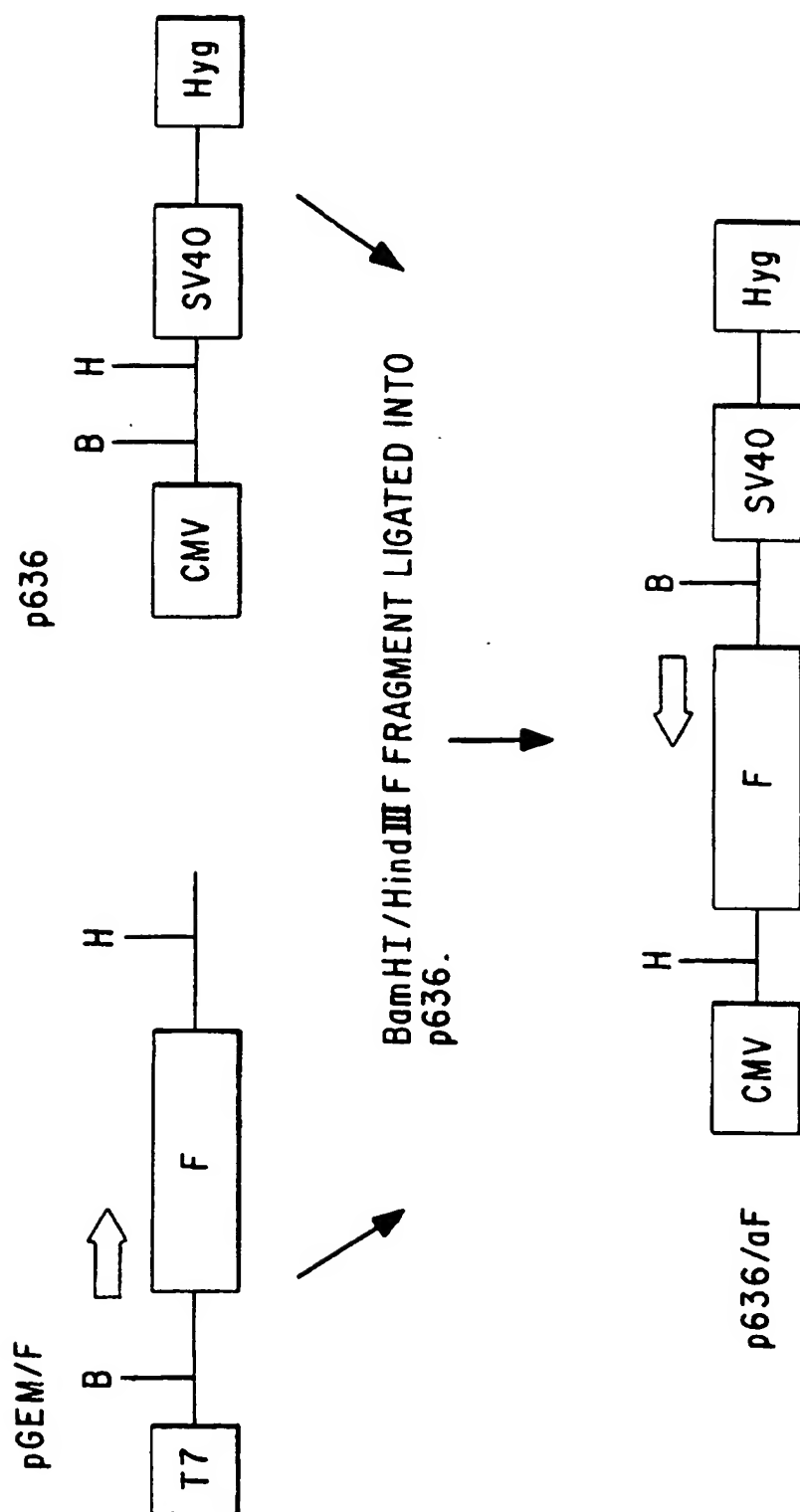


FIG. 1a

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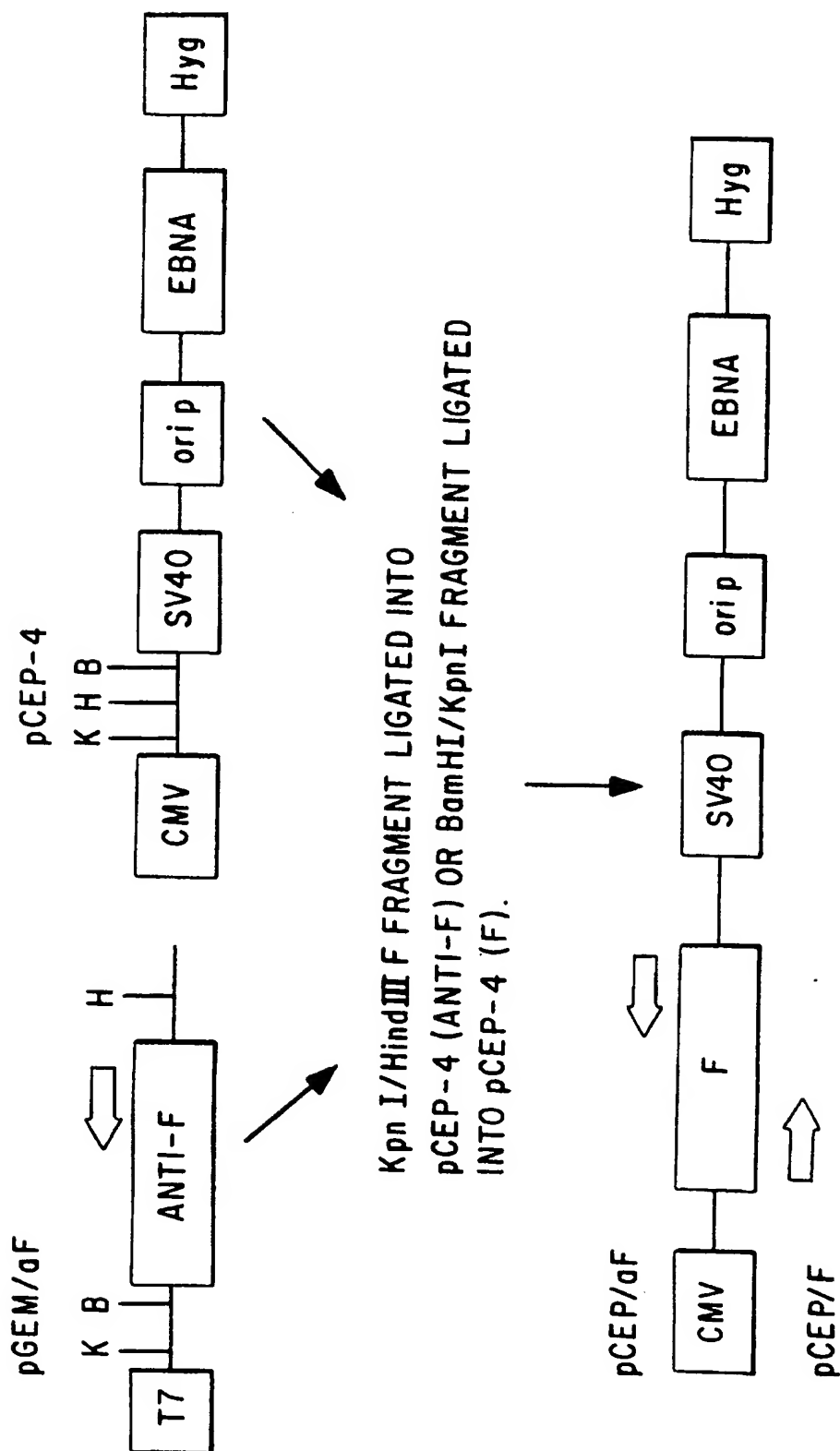


FIG. 1b

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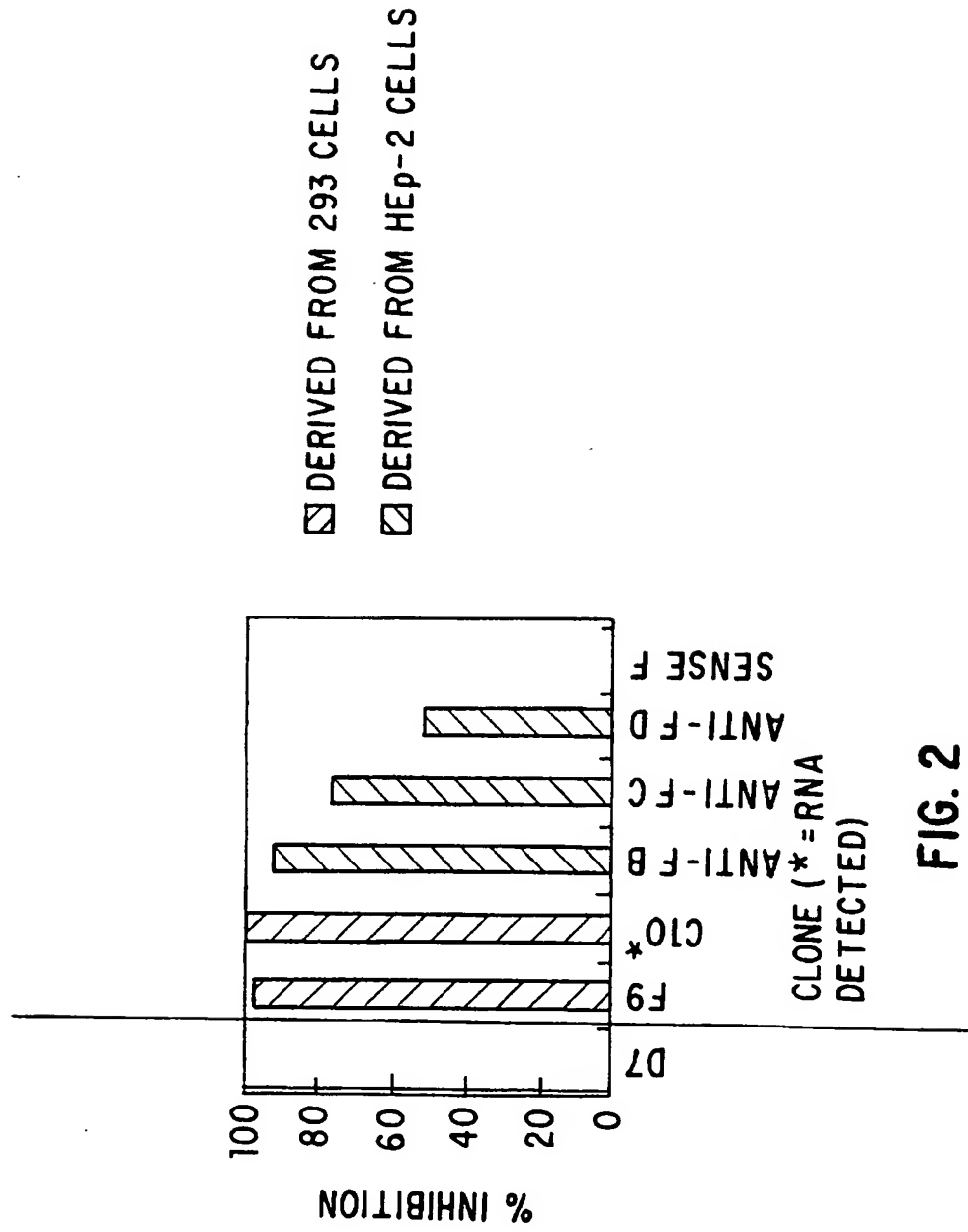


FIG. 2

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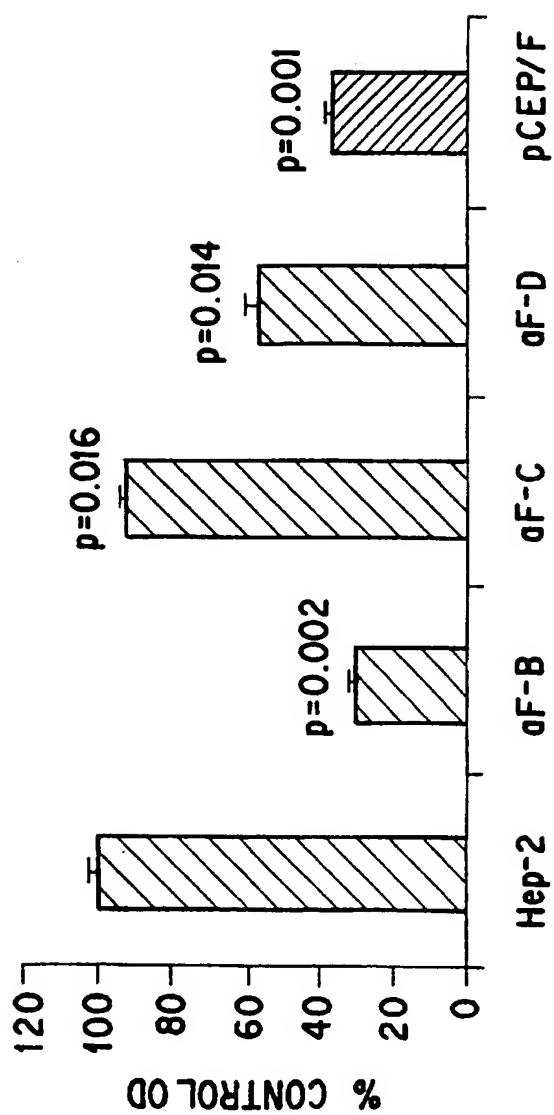


FIG.3a

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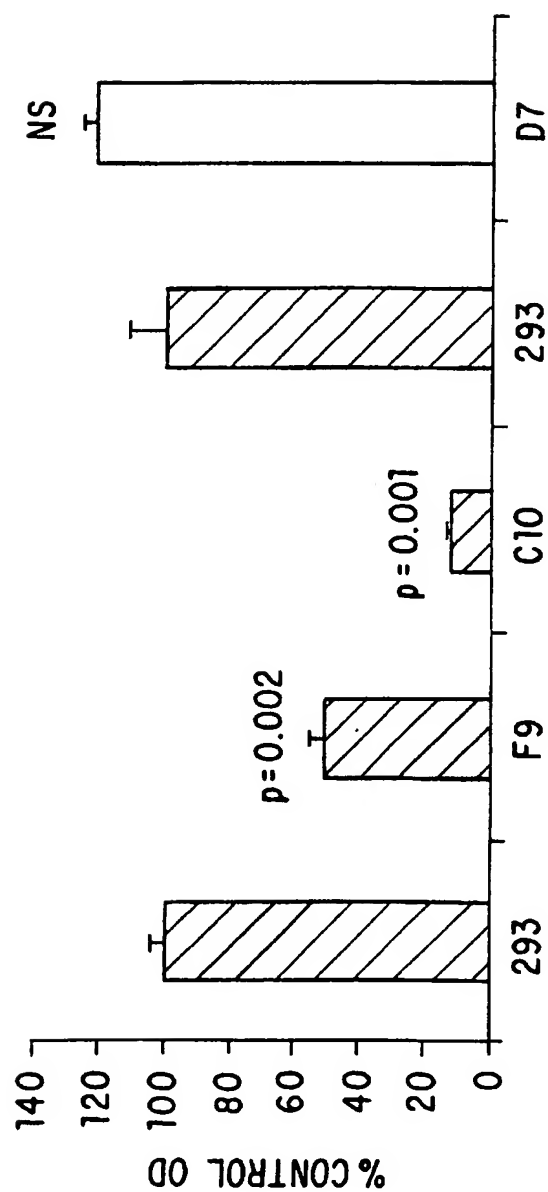


FIG. 3b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/16628

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/00, 5/10; A61K 31/395

US CL : 435/320.1, 325.1; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 325.1; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	THIERINGER, H.A. et al. Antisense RNA-Mediated Inhibition of Mouse Hepatitis Virus Replication in L2 Cells. Antisense Research and Development. 1995. Vol. 5. pages 289-294, see entire document.	1, 2, 9, 11, 13, 14, 17, 22, 23, 25, 28, 33, 34, 36 ----- 3, 8, 10, 19, 20, 22, 30, 31, 33

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 29 DECEMBER 1997	Date of mailing of the international search report 23 JAN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARY E. MOSHER, PH.D. Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/16628

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	MIZUTANI, T. et al. Both Antisense and Sense RNAs against the Nucleocapsid Protein Gene Inhibit the Multiplication of Mouse Hepatitis Virus. Journal of Veterinary Medical Science. 1994. Vol. 56. No. 5. pages 211-215, see entire document.	1, 2, 9, 11, 13, 14, 17, 22, 23, 25, 28, 33, 34, 36 ----- 3, 8, 10, 19, 20, 22, 30, 31, 33
Y	JALANKO, A. et al. An EBV-based mammalian cell expression vector for efficient expression of cloned coding sequences. Biochimica et Biophysica Acta. 1988. Vol. 949, pages 206-212, see entire document.	3, 8, 10, 19, 20, 22, 30, 31, 33
A	HELENE, C. et al. Specific regulation of gene expression by antisense, sense, and antigene nucleic acids. Biochimica et Biophysica Acta. 1990. Vol. 1049. pages 99-125.	1-36

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/16628

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, MEDLINE. SEARCH TERMS: ENUCLEAT?, PLANT, PLANTS, VIRUS, VIRAL, REPLICATION, REPLICATES, CYTOPLASM?, CYTOPLASMIC, TOBACCO(W)MOSAIC, ANTISENSE, INFLUENZA, ANTI(W)SENSE, VESICULAR(W)STOMATITIS, VSV, NUCLEUS, NUCLEAR, POLIO?, PICORNA?, RESPIRATORY(W)SYNCYTIAL, RSV, RNA(W)VIRUS?, TICK(1W)ENCEPHALITIS